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Characterization of Ryudocan Glycosaminoglycan Acceptor Sites*

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The specificity of the glycosaminoglycan (GAG) acceptor sites of ryudocan was examined by stably expressing epitope-tagged ryudocan cDNA constructs in mouse L cells, which normally produce this proteoglycan. Immunopurified ryudocan was glycanated with both heparan sulfate (HS) and chondroitin sulfate (CS). The attachment of GAGs to ryudocan was prevented by creating Ser → Thr mutations in all possible combinations at positions 44, 65, and 67. The resulting ryudocan of exogenous origin was immunopurified and evaluated with regard to attached GAG chains. The data reveal that ryudocan possesses three functional GAG attachment sites, that the sites are always occupied with GAG chains, and that each site is capable of bearing either HS or CS. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of GAG lyase digests of intact ryudocan reveal the production of the following multiple isoforms: pure HS-ryudocan, various HS/CS-hybrids, and pure CS-ryudocan. The data suggest that the occupancy bias of each site for HS or CS is slight and that each site functions in a relatively independent fashion. The GAG lyase analysis of partially purified L cell proteoglycans shows two pure CS-homoglycans with core proteins of M_r = 130,000 and 52,000, respectively. A similar analysis of immunopurified L cell glypican demonstrates that this species only exists as a pure HS-homoglycan. The production of pure homoglycans by this clonal cell line strongly suggests that the functional promiscuity of GAG attachment sites of ryudocan must be conferred in the core protein structure. This property of ryudocan is not peculiar to L cells, as ryudocan synthesized by early passage human endothelial cells also bears both HS and CS. The production of multiple isoforms of ryudocan may serve to expand the functional versatility of this cell surface component and allow it to participate in many different biologic processes.

Proteoglycans (PGs)[1] are a structurally diverse class of core proteins to which are coupled one or more glycosaminoglycans (GAGs) (for review see Ref. 1). The two major types of GAGs encountered are heparan sulfate (HS) and chondroitin sulfate (CS). The synthesis and placement of these GAGs on protein cores appear to be regulated by both the structure of the core protein and the posttranslational modification machinery.

Production of both HS and CS is thought to begin in the late endoplasmic reticulum and *cis*-Golgi with the addition of a common linkage tetrasaccharide to particular serine sites of the core proteins (for review see Refs. 2–4). The biosynthetic pathways then diverge with the generation of a linear backbone of [GlcUA(β1,4)→GlcNAc(α1,4)→]$_n$ for HS or [GlcUA(β1,3)→GalNAc(β1,4)→]$_n$ for CS. The respective copolymer chains are then variably modified by pathway specific epimerization and sulfation reactions (for review see Ref. 1).

The biochemical analysis of cell mutants strongly suggests that an identical set of enzymes is responsible for the synthesis of the linkage region common to both HS and CS (5–7). Thus, it appears likely that commitment to place HS or CS on core proteins must occur at the initiation of copolymer elongation, with the introduction of the first hexosamine residue (GlcNAc for HS, or GalNAc for CS). Elongation of HS and CS may be accomplished, respectively, by two distinct copolymerases that possess both glucuronosyl- and *N*-acetylhexosaminyltransferase activities (8). The relative activities of these two polymerases may partially determine the number of HS and CS chains coupled to specific protein cores (9), or alternatively, posttranslational modification of linkage region residues (10) may regulate the specificity of initiation. However, the structure of the protein core must also be involved in determining the specificity of GAG attachment since certain PGs bear exclusively HS or CS despite a late Golgi environment that is capable of generating both GAG types (11–13).

The involvement of core structure is less clear for hybrid PGs such as serglycin (for review see Ref. 14) and syndecan (15), which possess both HS (or structurally similar heparin) and CS. These two core proteins exhibit a large number of potential GAG acceptors sites, and multiple glycanation isoforms are produced. Serglycin exists as a CSPG, an HPG, or a mixed CSPG/HPG in different cell types (16–18). For syndecan, the number of HS and/or CS chains/core protein is modulated by differentiation (19) and by transforming growth factor-β (20). It is unclear whether this structural diversity results from the presence of HS-specific and CS-specific acceptor sites or if both GAG types can be attached to acceptor sites. Thus, the determination of the type of GAG attached to individual acceptor sites in a hybrid PG is important to our understanding of how protein core structure collaborates with the rough endoplasmic reticulum/Golgi machinery to define the placement of HS or CS.

We have recently isolated the cDNA for rat endothelial cell ryudocan (21). Ryudocan is a member of the syndecan family of

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[1] The abbreviations used are: PG(s), proteoglycan(s); GAG(s), glycosaminoglycan(s); HS, heparan sulfate; CS, chondroitin sulfate; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropy)dimethylammonio]-1-propanesulfonic acid; HPG, heparin proteoglycan; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MT, metallothionein.

cell surface PGs (syndecan-4) (22), which suggests that it may possess HS and CS. In this investigation, we confirm this supposition by expressing an epitope-tagged ryudocan cDNA in a clonal L cell line, as well as primary endothelial cells, and then determine the types of GAGs attached to the core protein. Furthermore, using site-directed mutagenesis, we identify all three functional GAG attachment sites and ascertain the types of GAG chains present at each site. The results show that HS and CS chains are covalently linked to each of these sites in a relatively unbiased manner and that the three acceptor sites function in a relatively independent fashion. These data suggest that hybrid proteoglycans may contain promiscuous GAG attachment sites that allow for the generation of multiple, structurally diverse, and functionally discrete isoforms.

EXPERIMENTAL PROCEDURES

Cell Culture and Endothelial Cell Isolation—A clonal L cell line (23) was grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum at 37 °C under 5% CO_2 humidified atmosphere. The cells were maintained in logarithmic growth by subculturing biweekly with conditioning to this regimen for at least 6 weeks prior to experimental analysis. To ensure consistency, analysis was performed on cultures that were confluent (~21 × 10^6 cells/75-cm^2 flask) for 7 days. Postconfluent L cell cultures were generated by inoculating 75-cm^2 flasks with 20 × 10^6 cells on day 0, daily feeding (25 ml of medium) was initiated on day 3, and cultures were radiolabeled and harvested on day 7 when the final density was ~60 × 10^6 cells/flask. Transfected cells grew 10–15% slower than nontransfected cells; therefore, inocula were adjusted to achieve the above final density.

Endothelial cells were prepared from residual saphenous vein segments of elective coronary bypass patients (approved by Brigham and Women's Hospital's Human Institutional Review Board as use of otherwise discarded material). Veins were transported in chilled heparinized electrolyte solution (Plasma-lyte, Baxter Health Care, Detroit), and endothelial cells were enzymatically harvested (24–26). In brief, cannulated segments were flushed with Hanks' balanced salt solution containing Ca^{2+} and Mg^{2+} (HBSS$^+$, Life Technologies, Inc.) at 37 °C for 15 min while gently distended with HBSS$^+$ containing 0.1% collagenase (Type II, Worthington) and 0.1% bovine serum albumin. Endothelial cells were flushed from segments with M-199 medium (Life Technologies, Inc.) and then pelleted by centrifugation for 5 min at 125 × *g*.

The cells were cultured on 1% gelatin-coated dishes in M-199 supplemented with 25 mM HEPES, 20% fetal bovine serum (HyClone, Logan, UT), 0.7 mM L-glutamine, 50 units/ml penicillin G, 50 μg/ml streptomycin sulfate, 125 ng/ml amphoteredin B, 17.5 units/ml (~100 μg/ml) sodium heparin (Sigma), and 100 ng/ml endothelial cell mitogen (Biomedical Technologies, Inc., Stoughton, MA) at 37 °C under 5% CO_2. Medium was changed three times weekly, and at confluence cells were subcultured at a split ratio of 1:6. Endothelial cell phenotype was confirmed by the presence of factor VIII-related antigen (27) and scavenger receptor (28). Factor VIII-related antigen was detected by indirect immunohistochemical staining with a primary goat antibody (Atlantic Antibodies, Scarborough, ME) and secondary avidin-biotin-peroxidase detection (Vector Laboratories, Burlingame, CA). Scavenger receptor was detected as uptake of acetylated low density lipoprotein tagged with the fluorescent dye 1,1'-dioctadecyl-1–3,3,3',3'-tetramethylindocarbocyanine perchlorate (Biomedical Technologies Inc.) (28). Smooth muscle cell contamination was excluded by the absence of α-actin. Indirect immunohistochemical staining for actin isoforms employed the HHF 35 murine monoclonal antibody (HHF 35, Enzo Biochemicals, Inc., New York) (29).

Plasmid and Retrovirus Constructions—The G418 resistance vector, pNWS61, was constructed similar to pMEV39R (30) except that a human MT-II_A promoter (−761 to +70) (31) was used to drive the bacterial *neo*^{r} gene. The ryudocan expression plasmid pNWS144 (Fig. 1*A*) was derived from the vector pNWS96 (21). First, the 12CA5 epitope was created at the carboxyl terminus of the ryudocan coding sequence (Fig. 1*B*) with polymerase chain reaction mutagenesis (32) by inserting the codons for PYDVPDY after codon 201. Then, the ryudocan coding sequence was flanked upstream by the human MT-II_A promoter (an *Sst*I/*Bam*HI fragment from pNWS61) and downstream (nucleotide 628, blunted *Hin*dIII) by the human growth hormone gene poly(A) signal (nucleotides 1530–2155, blunted *Bgl*I to *Eco*RI) (33). Polymerase chain reaction mutagenesis was also employed to create seven derivatives of pNWS144 in which serines 44, 65, and 67 are converted to threonine in all possible combinations (Fig. 1*C*, pNWS155–pNWS162). Sequencing confirmed that all mutagenized regions possessed the anticipated DNA sequences.

A replication defective retroviral vector expressing carboxyl-terminal tagged ryudocan was created by first cloning the ryudocan$_{12CA5}$ coding region of pNWS144 (*Nco*I to *Hin*dIII) into a vector backbone (MFG) (34). Supernatant containing ψCRIP packaged (amphotropic) transducing particles was generated and harvested as described previously (35). In brief, producer cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 50 units/ml penicillin G, and 50 μg/ml streptomycin sulfate. Supernatants containing recombinant retrovirus were prepared by exchanging producer cell medium 24 h prior to confluence. At confluence, medium was harvested, filtered (0.45-μm Acrodisc, Gelman, Ann Arbor, MI), supplemented with 32 μg/ml DEAE-dextran (Sigma), and used that day for transduction.

Stable Cotransfections and Retroviral Transductions—L cells (10^6/100-mm plate) were grown for 1 day, and then 1 ml of $Ca_3(PO_4)_2$-DNA precipitate (36) was added. The precipitate contained 15 μg of ryudocan expression construct, 0.5 μg of the G418 resistance vector pNWS61, and 15 μg of sheared herring sperm DNA. After 15 h, cells were refed, and 4 days later antibiotic-resistant cells were selected with 400 μg/ml G418 (Life Technologies, Inc.). Selective pressure was removed 2 weeks after cultures became confluent. At this time, the heterogeneous pools were either analyzed or cloned by limiting dilution.

For retroviral transduction of endothelial cells, monolayers (16 h postinoculation) were washed with PBS and then incubated overnight in filtered retrovirus supernatants. The following day, the supernatant was replaced with a second fresh viral stock and incubated for 16 h. The confluent monolayers (transduced and nontransduced controls) were then trypsinized and frozen in aliquots.

Analysis of Expression Constructs—*In vitro* transcription/translation analysis was performed as described previously (21). To examine ryudocan expression levels in transfected cells, cytoplasmic RNA was isolated (30), and 30-μg samples were subjected to dot-blot (37) and Northern blot (21) analyses. Samples were hybridized to a ^{32}P random primed ryudocan ectodomain probe (nucleotides 219–447) in 1 M NaCl, 50% formamide, 1% SDS, and 10% dextran sulfate (molecular weight ~500,000) at 42 °C overnight. Membranes were washed twice in 2 × SSC, 0.1% SDS for 10 min at 24 °C and then twice at 0.4 × SSC, 1% SDS for 30 min at 65 °C. Levels of hybridized probe were measured with a Betascope 603 Blot Analyzer (Betagen, Waltham, MA).

Metabolic Labeling of Proteoglycans—To radiolabel L cell GAGs, cultures were washed twice with PBS (5 ml) and then incubated for 1 h with 4 mCi of $Na_2{}^{35}SO_4$ (carrier-free, ICN) in 2 ml of a modified basal medium of Eagle (Life Technologies, Inc.) supplemented with 1% Nutridoma-SP (Boehringer Mannheim) and equilibrated with 5% CO_2. This medium was altered from the basal medium of Eagle composition by substituting $MgSO_4$ with equimolar $MgCl_2$, reducing NaCl to 100 mM, and adding 1 mM L-glutamine, 1 mM sodium pyruvate, and 25 mM HEPES (pH 7.4). For determinations of GAG synthetic rate, this medium was adjusted to 400 μM Na_2SO_4, which maximizes cellular uptake of sulfate. Conversely, 20 μM Na_2SO_4 was used for immunoprecipitations and PG analysis to maximize the specific activity of labeling. For L cell protein core labeling, PBS-washed cultures were preincubated for 1 h in either leucine-free or methionine-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and then incubated for 1 h in 2 ml of medium containing 1 mCi of L-[3,4,5-^{3}H]leucine (166 Ci/mmol, DuPont NEN) or 3 mCi of L-[^{35}S]methionine (693 Ci/mmol, DuPont NEN), respectively. To generate PG-enriched cell extracts, labeled monolayers were washed six times with ice-cold PBS, overlaid with 0.75 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM iodoacetate, 100 μM phenylmethylsulfonyl fluoride, 0.02% NaN_3), and incubated on ice for 20 min. Lysates were collected and pooled with a subsequent monolayer rinse (0.5 ml of lysis buffer). Lysates were then centrifuged at 15,000 × *g* for 10 min at 4 °C, and supernatants were snap frozen with liquid N_2 and subsequently analyzed. Cell extract protein concentration was determined by the procedure of Bradford (38) using BSA (Sigma A7906) as a standard. Frozen transduced endothelial cells were thawed, passaged once, and 2-day postconfluent cultures were radiolabeled as described above except that the medium was supplemented with 0.05% human serum albumin (Immuno-U.S., Rochester, MN), and monolayers were collected in 0.5 ml of lysis buffer.

Quantitation of GAG Synthesis—^{35}S incorporation into total PG GAGs was measured by analytical DEAE chromatography, described below. To determine HS and CS synthetic rates, preparative DEAE chromatography was used to purify total PGs from which total GAGs were isolated. The relative abundance of HS and CS was then measured

using appropriate GAG lyases. Synthetic rates were calculated as (^{35}S incorporation into total PG GAGs) × (relative abundance).

DEAE Chromatography—For analytical DEAE chromatography, cell extracts (100 μg) were adjusted to 291 μl with lysis buffer, mixed with 750 μl of PLB (222 mM NaCl, 69.4 mM sodium acetate (pH 5.0), 1.39 mM EDTA, 0.83% CHAPS (Sigma), 8.33 M urea, 1.39 mM Na_2SO_4, and 0.03% NaN_3), and then charged to 160-μl beds of DEAE-Sepharose Fast Flow (Pharmacia Biotech. Inc.) by two successive passes. Columns were washed with 2 ml of UPAS (150 mM NaCl, 50 mM sodium acetate (pH 5.0), 1 mM EDTA, 0.6% CHAPS, 6 M urea, 1 mM Na_2SO_4, 0.02% NaN_3), and PGs were eluted with 1.5 ml of 1 M NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.6% CHAPS, 6 M urea, and 0.02% NaN_3. The eluate was subjected to liquid scintillation counting. This procedure was quantitative in that all GAG lyase-digestible radioactivity was removed from the cell extract, >98% of DEAE bound radioactivity was recovered, and >99.5% of the resulting ^{35}S material was digestible by the combined activities of *Flavobacterium* heparitinase and chondroitinase ABC.

Preparative DEAE chromatography was similarly performed except for the following. One to 6 mg of cell extract was charged to DEAE columns; columns were washed with 4 ml of UPAS; 2 ml of 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.6% CHAPS, 0.02% NaN_3; 2 ml of 150 mM NaCl, 0.03% Triton X-100, 30 μg/ml BSA); and then eluted with 1 ml of 1 M NaCl, 0.03% Triton X-100, 30 μg/ml BSA, with 30 ng/ml dextran sulfate (Sigma, molecular weight ~8,000) as carrier.

GAG Isolation and Analysis—The GAGs were cleaved from PGs by β-elimination. 10 μl of 6.58 M NaOH containing 0.89 M $NaBH_4$ was added to the 1 ml of preparative DEAE eluate, and after a ~16-h incubation at 46 °C, the reaction was quenched with 60 μl of 8.54 M ammonium formate containing 1.7 M HCl. Samples were chilled on ice and extracted twice against 0.75 ml of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous volume was reduced to 100 μl by SpeedVac (Savant), samples were chilled on ice for 15 min, and salt was removed by centrifugation for 2 min at 10,000 × *g*. The supernatant was adjusted to 350 μl with water, and GAGs were precipitated by the addition of 1.25 ml of absolute ethanol followed by centrifugation at 15,000 × *g* for 20 min at 4 °C. The pellet was resuspended in 200 μl of 0.0005% Triton X-100 followed by removal of residual ethanol by incomplete SpeedVac concentration. Analysis with *Flavobacterium* heparitinase and chondroitinase ABC demonstrated that the ratio of HS to CS of purified GAGs was the same as the source PG population.

The relative content of HS and CS was determined as ethanol-soluble ^{35}S counts generated by the respective activities of *Flavobacterium* heparitinase and chondroitinase ABC. A 110-μl digestion stock contained total GAGs (~10,000 cpm), 10 μg of BSA, 10 μM dextran sulfate (molecular weight ~8,000), 30 mM NaCl, and 25 mM ammonium acetate (pH 7.0). To 49-μl portions of this mixture was added either 1 μl of 20 mg/ml chondroitin sulfate (Sigma, C-4384) with 2 μl (0.46 unit) of *Flavobacterium* heparitinase (prepared as described previously) (39) or 3 μl (0.018 unit) of chondroitinase ABC (Sigma, C-2905). Reactions were incubated for 2.5 h at 43 or 37 °C, respectively. Then, 10 μl of 1 M NaCl and 150 μl of ethanol were added, and samples were centrifuged for 20 min at 15,000 × *g*. The supernatant (150 μl) was then subjected to liquid scintillation counting. In the absence of enzyme, <0.3% of total GAG was ethanol-soluble, whereas in the presence of both enzymes, the entire GAG sample was converted into ethanol-soluble counts.

For analysis of GAG chain size, HS and CS samples were prepared from total GAGs by digestion with either chondroitinase ABC or *Flavobacterium* heparitinase, respectively, in 100-μl reactions as described above. After digestion, reactions were extracted against 300 μl of phenol/chloroform/isoamyl alcohol (25:24:1); samples were precipitated by adding 10 μl of 1 M NaCl, with 500 μl of absolute ethanol, and centrifuged for 20 min at 15,000 × *g* at 4 °C. Pellets were resuspended in 20 μl of SDS sample buffer and resolved by 15% SDS-PAGE (42) along with 4 nCi of ^{14}C-methylated protein markers (Amersham Corp.). Migration profiles were recorded with a Betascope 603 Blot Analyzer. To obtain the M_r values of GAGs, the protein markers were calibrated against CS size standards having modal molecular masses of 14,100 and 36,000 (supplied and characterized by Åke Wasteson) (40) and 20,000 Da (a gift from Vincent Hascall, NIH) (modal GAG M_r = 2.112 × protein M_r − 21027, r = 0.934).

Immunoprecipitation of Ryudocan and Glypican—All immunoprecipitation steps were performed on ice or at 4 °C. To isolate epitope-tagged ryudocan, cell extract (600 μl) was mixed with 16 μl of 10% SDS, 10 μg of normal mouse IgG, and 200 μl of NET (50 mM Tris (pH 7.4), 150 mM NaCl, 0.25% gelatin, 0.1% Triton X-100, 1 mM EDTA, 0.02% NaN_3) and incubated for 1 h. Upon adding 30 μl of NET equilibrated (1:1 slurry) protein A-Sepharose (Pharmacia Biotech. Inc.), samples were agitated for 1 h. After centrifugation for 10 min at 15,000 × *g*, the precleared supernatants were added to 57 μg of protein G-purified 12CA5 monoclonal antibody (Harvard Monoclonal Antibody/Cell Culture Facility) and incubated for 1 h. The antibody-bound proteins were then captured with protein A-Sepharose/NET (160 μl) by incubation for 1 h with agitation. Beads were collected by centrifugation for 10 s at 8,000 × *g*, and when required the supernatant was subjected to additional cycles of 12CA5 incubation/protein A-Sepharose extraction. Each cycle removed ~50% of recoverable material from the cell extract. Beads were pooled and washed in 1.25 ml of NET containing 0.1% SDS and then 1.25 ml of NET adjusted to 0.5 M NaCl. For SDS-PAGE analysis, beads were washed with 1.25 ml of 10 mM Tris (pH 7.4) and 0.1% Triton X-100. Bound proteins were eluted by incubating beads in 40 μl of SDS sample buffer at 95 °C for 10 min. Alternatively, beads were washed twice with BDT (0.1 mg/ml BSA, 10 μM dextran sulfate, 3.3 mM Tris (pH 7.4), 0.33% Triton X-100, 0.02% NaN_3). Tagged ryudocan was then eluted by three sequential 100-μl incubations in BDT containing a 24 μM concentration of the 12CA5 epitope peptide YPYDVPDYA (Berkeley Antibody Co., Inc.). The intact PG was then either subjected to GAG lyase digestion, or GAGs were removed by β-elimination and then purified and analyzed as described above for total PGs.

Rabbit antiserum was raised against rat ryudocan ectodomain or intracellular domain peptides (described below) conjugated to keyhole limpet hemocyanin (peptide and antiserum generated by Immuno-Dynamics Inc., La Jolla, CA). For analysis of endogenous ryudocan, preimmune serum was substituted for mouse normal IgG, whereas 12CA5 monoclonal antibody was replaced with a rabbit polyclonal antiserum (40 μl). Purification of glypican was similarly performed with anti-glypican rabbit serum (41) (a gift from Arthur Lander, MIT) raised against a bacterially generated glypican/maltose-binding protein fusion product. To ensure that cell extracts contained glypican, 0.5% CHAPS was included in the lysis buffer. GAG lyase reactions were performed on bead-bound material, and samples were subsequently eluted with SDS sample buffer, as above, and analyzed by SDS-PAGE.

Analysis of Endogenous Proteoglycan Cores—Cell extract (700 μg) was adjusted to 770 μl with cell lysis buffer, then mixed with 1.2 ml of GUTE (4 M guanidine HCl, 50 mM Tris (pH 7.4), 1 mM EDTA), and subjected to ultrafiltration (Centricon-100, Amicon). The buffer was exchanged twice with GUTE, twice with PBS containing 1% glycerol, and then once with H_2O. The retentate was adjusted to 590 μl with lysis buffer, mixed with 1.52 ml of PLB, and subjected to preparative DEAE chromatography as described above. The resulting eluate was mixed with 1 ml of GUTE and applied to a Centricon-100 ultrafilter. The buffer was exchanged once with GUTE and twice with water. The retentate was then subjected to GAG lyase digestion and ethanol precipitation, as described above for GAGs. Pellets were resuspended in 20 μl of SDS sample buffer, heated to 95 °C for 15 min, and analyzed by 3–18% linear gradient SDS-PAGE.

SDS-PAGE and Generation of Migration Profiles—SDS-PAGE was performed as described previously (42). Resolved samples were visualized by autoradiography or fluorography using EnHance (DuPont NEN). Migration profiles were obtained with a Betascope 603 Blot Analyzer. Data were collected for 12–48 h at the maximal resolution setting, and migration was measured over 0.4-mm increments. M_r was determined by calibration against ^{14}C-methylated protein markers (4 nCi/lane) (Amersham Corp.). Curve fitting analysis was performed on a 486 based personal computer using the software Origin (MicroCal Software, Inc.; Northampton, MA).

Analysis and Comparisons of cDNA Sequences—Sequence analyses were performed with the University of Wisconsin Genetics Computer Group sequence analysis software package. Sequence comparison searches were performed against GenBank, GNEW, EMBL, and NBRF data bases. RNA secondary structure was predicted with the program "Fold."

RESULTS

Experimental Strategy: Overexpression of Epitope-tagged Ryudocan—We determined the relative amount of HS and CS attached to the various GAG acceptor sites of ryudocan by stably expressing the rat cDNA in a clonal mouse L cell line that normally synthesizes this PG. Additional codons were inserted at the carboxyl terminus of the ryudocan coding sequence to create a unique epitope tag (Fig. 1*B*), the influenza virus hemagglutinin epitope (YPYDVPDYA) (43), which is recognized by the 12CA5 monoclonal antibody (44). This alteration allows the exogenous origin ryudocan bearing the 12CA5 epitope (designated as ryudocan$_{12CA5}$) to be specifically isolated

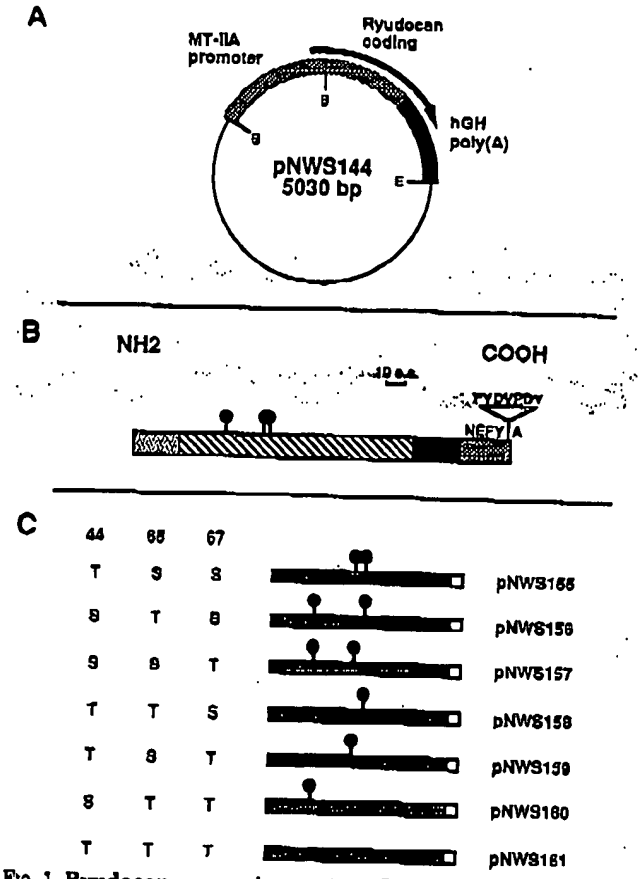

FIG. 1. **Ryudocan expression vectors.** *Panel A*, as described under "Experimental Procedures," the ryudocan$_{12CA5}$ cDNA coding region (*cross-hatched*) was expressed with vector pNWS144 or its derivatives. Relevant restriction sites are displayed: *BamHI* (*B*), *EcoRI* (*E*), and *SetI* (*S*). Ryudocan message is transcribed by the human metallothionein II_A promoter (*MT-IIA*) and stabilized by 3′ poly(A) signals obtained from the human growth hormone (*hGH*) gene. *Panel B*, ryudocan is a type I integral membrane protein composed of an amino-terminal leader sequence (*stippled*), an ectodomain (*striped*) containing three GAG attachment sites (*ball* and *stick* structures), a transmembrane domain (*solid black*), and a carboxyl-terminal intracellular domain (*cross-hatched*). The carboxyl-terminal 12CA5 epitope (YPYDVPDYA) is composed of 7 inserted codons and the 2 flanking ryudocan residues. The inserted amino acids as well as the flanking residues are indicated. *Panel C*, derivatives of pNWS144 all contain the carboxyl-terminal epitope tag and one or more threonines (*T*) at positions 44, 65, and 67 to destroy GAG acceptor serines (*S*). The specific residues of each construct at these positions and a schematic representation of the resultant PGs are displayed.

FIG. 2. **Ryudocan overexpression elevates the synthesis of both HS and CS.** Nontransfected L cell and clone 19 (transfected with pNWS144) cultures were Na$_2$^{35}SO$_4$ labeled for 1 h. GAGs were isolated, and ^{35}S incorporation into HS and CS was enzymatically determined as described under "Results." Results from duplicate samples are presented (mean ± range).

by immunoprecipitation.

All employed expression plasmids were derived from pNWS144, which contains the ryudocan$_{12CA5}$ coding region transcribed by the high basal activity (45) of the human MT-II_A promoter (Fig. 1A). Control experiments demonstrated that immunoprecipitation of ryudocan$_{12CA5}$ was most efficient when the 12CA5 epitope was localized to the carboxyl terminus, as opposed to localization after the amino-terminal leader sequence or before the transmembrane domain (results not shown). Chou-Fasman analysis (46) predicts an absence of discrete secondary structure at this insertion site. The insertion had no effect on membrane translocation of the protein core or leader peptide cleavage, as confirmed by subjecting *in vitro* synthesized transcripts to *in vitro* translation in the absence and presence of microsomes (data not shown) (21). Thus, insertion of the epitope should not affect steps prior to core protein glycanation. These experiments also revealed that addition of the epitope tag increases the apparent M_r of the amino-terminal processed core protein from 33,000 to 35,000.

Ryudocan Is a Hybrid Proteoglycan—To determine the type of GAGs attached to ryudocan, L cells were stably transfected with pNWS144, and clones generated by limiting dilution were screened for high ryudocan expression by Northern and dot-blot analyses. We present data from a representative clone, clone 19, which expresses 33-fold greater ryudocan mRNA than the nontransfected L cell clone, as measured by dot-blot analysis. However, the examination of four additional clones with equivalent or greater expression levels produced data similar to that provided below.

Cultures of the nontransfected L cell and clone 19 were radiolabeled for 1 h with Na$_2$^{35}SO$_4$. Triton X-100-soluble cell extracts were prepared, and PGs were isolated by DEAE chromatography. The GAGs were liberated from PGs by β-elimination in the presence of sodium borohydride and then isolated by ethanol precipitation. ^{35}S incorporation into HS or CS was then determined as ethanol-soluble radioactivity generated after digestion with *Flavobacterium* heparitinase or chondroitinase ABC, respectively (as described under "Experimental Procedures"). The 1-h labeling period used should detect alterations of PG synthesis relatively independent of catabolism, given that the half-life for turnover of cell surface intercalated PGs typically ranges from 3 to 8 h (for review see Ref. 47). Clone 19 exhibited increased ^{35}S incorporation into HS (1.7-fold) and CS (1.3-fold) relative to nontransfected cells (Fig. 2). Similarly, with [^{3}H]glucosamine labeling of cells, HS and CS levels were found to be increased by 1.5- and 1.2-fold, respectively. These results suggest that ryudocan$_{12CA5}$ is glycanated and bears both HS and CS.

Clone 19 exhibits a relatively small increase in GAG synthesis as compared with the large augmentation in ryudocan mRNA levels. This observation implies that endogenous ryudocan comprises a small fraction of total PGs and/or that the

recombinant mRNA exhibits a low translational efficiency relative to the endogenous species. To examine this issue, we performed exhaustive immunoprecipitation on nontransfected cell extracts with rabbit polyclonal antiserum raised against the intracellular domain peptide epitope CLGKKPIYKKAPTNE (cysteine plus residues 186–199).[2] Two sequential immunoprecipitation cycles extracted 15 ± 1% of GAG cpm from nontransfected cell extracts (n = 9), whereas the addition of the peptide CLGKKPIYKKAPTNE reduced recovery of radioactivity to essentially zero. Immunoprecipitation with polyclonal antibodies can underestimate antigen abundance (48), thus these results indicate that endogenous ryudocan accounts for a substantial level of GAG synthesis in nontransfected L cells. Accordingly, it seems possible that the recombinant ryudocan mRNA may exhibit a low translational efficiency. Consistent with this suggestion, the theoretical analysis of the structure of the recombinant message (49) predicts a stable 5′ hairpin, which has been noted to reduce translational efficiency greatly (50).

We then isolated ryudocan$_{12CA5}$ from clone 19 by 12CA5 immunopurification, purified total PGs from nontransfected L cells by DEAE chromatography, and immunofractionated endogenous ryudocan from nontransfected L cells with the antiserum specific to the intracellular domain, as outlined above. The attached GAGs were isolated, and the relative amounts of HS and CS were enzymatically determined, as described previously. The exogenous origin ryudocan possesses 87% of GAG ^{35}S as HS and the remaining 13% as CS, which is virtually identical to the distribution observed for endogenous ryudocan or total PGs produced by nontransfected cells (Fig. 3). Thus, the GAG composition of ryudocan is not biased by overexpression of epitope-tagged core protein. Therefore, this approach provides a valuable means of determining if HS or CS is attached to individual GAG acceptor sites. Such analysis first requires an identification of all possible GAG acceptor sites on ryudocan.

Identification of Ryudocan GAG Acceptor Sites—Ryudocan exhibits three potential GAG acceptor sites (Ser-Gly in the vicinity of acidic residues) at serine residues 44, 65, and 67. Previous investigators have demonstrated with other PGs that mutation of the GAG acceptor serine to threonine dramatically reduces the attachment of GAG chains (12, 51). Therefore, we evaluated the function of the postulated GAG acceptor sites in ryudocan$_{12CA5}$ by creating plasmids with all seven possible combinations of Ser → Thr substitution (Fig. 1*C*) and used each plasmid to stably transfect L cells. The respective ^{35}S-labeled forms of ryudocan$_{12CA5}$ were isolated from each cell population and analyzed by SDS-PAGE (Fig. 4). The molecular size of ryudocan$_{12CA5}$ was proportional to the number of remaining putative GAG acceptor sites, independent of which specific serine residues were mutated. As intact potential acceptor sites decreased from three to zero, the apparent modal M_r of the respective molecules was 280,000, 210,000, 120,000 and 39,000. The last species is nominally larger than expected for the core protein and is designated as minimally modified ryudocan.

The detection of minimally modified ryudocan (Thr^{44,65,67} ryudocan$_{12CA5}$) with $Na_2{}^{35}SO_4$ is unexpected since the core protein lacks ^{35}S-containing GAGs. Secondary incorporation through amino acids could not have generated a sufficiently high specific activity to explain the above observation.[3] Thus, radiolabeling of the minimally modified core protein must have resulted from a sulfate containing posttranslational modification. Consistent with this suggestion, the minimally modified core protein is ~4,000 daltons larger than *in vitro* synthesized ryudocan$_{12CA5}$ protein (described above). Ryudocan lacks an appropriate consensus sequence for tyrosine *O*-sulfate (52, 53); however, the modification could be an asparagine-linked sulfated carbohydrate (54). Regardless, the moiety allows visualization of the minimally modified core protein with $Na_2{}^{35}SO_4$ labeling, which has been utilized in subsequent experiments (see below).

We note that deletion of each GAG acceptor site causes a

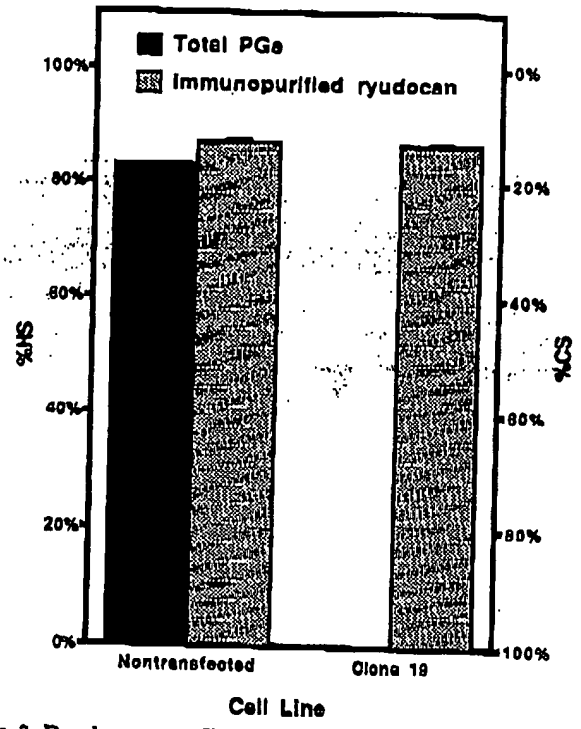

FIG. 3. **Ryudocan$_{12CA5}$ GAG composition is similar to endogenous ryudocan.** ^{35}S-Labeled GAGs were prepared from PG fractions of nontransfected cells, as well as ryudocan$_{12CA5}$ immunopurified from clone 19 with the 12CA5 antibody. For each sample of nontransfected cell extract, GAGs were prepared from total PGs and immunoprecipitated ryudocan, as described under "Experimental Procedures." The relative content of HS or CS was determined by digestion with *Flavobacterium* heparitinase or chondroitinase ABC, respectively (as described under "Results"). Results from duplicate samples (mean ± range) are presented, and similar data were obtained in two additional experiments.

[2] The ^{35}S-labeled material recovered by immunoprecipitation was PG, thus the specificity of this antibody was confirmed by Western blot analysis of core proteins purified from nontransfected L cells. DEAE-isolated PGs were digested with GAG lyases and analyzed. The electrophoretic patterns revealed a major (80%) species of M_r = 35,000, which corresponds to the anticipated size for the ryudocan core, and a minor (20%) species of M_r = 75,000, which is likely to be a dimer of the ryudocan core protein. Both components are also detected with antiserum generated against the rat ryudocan ectodomain peptide, CEPKELEENEVIPKP (cysteine plus residues 106–119).

[3] The mature, amino-terminal processed ryudocan core lacks cysteine, but potentially secondary incorporation through methionine may have occurred. To exclude this possibility, pNWS161-transfected cells were labeled for 1 h with $Na_2{}^{35}SO_4$ or [^{3}H]leucine. The relative incorporation into the ryudocan$_{12CA5}$ core was measured by 12CA5 immunoprecipitation and was standardized to the incorporation into non-PG species (determined as ethanol-precipitable cpm minus DEAE-bound cpm). If ^{35}S radioactivity incorporates through methionine, then the relative incorporation for ^{35}S and ^3H will be similar since labeling with any amino acid should approximate the contribution of Thr^{44,65,67} ryudocan$_{12CA5}$ toward total protein synthesis. Conversely, if ^{35}S incorporates by a specific posttranslational modification, then the relative incorporation of ^{35}S will exceed that of ^3H since most non-PG molecules should lack the postulated modification. ^{35}S- and ^3H-labeled Thr^{44,65,67} ryudocan$_{12CA5}$ comprised 0.17% and 0.00059%, respectively, of non-PG radioactivity. Given that mature ryudocan contains 2 methionine and 17 leucine residues, the specific activity of ^{35}S incorporation into Thr^{44,65,67} ryudocan$_{12CA5}$ was at least 2,400-fold greater than could have occurred through methionine incorporation.

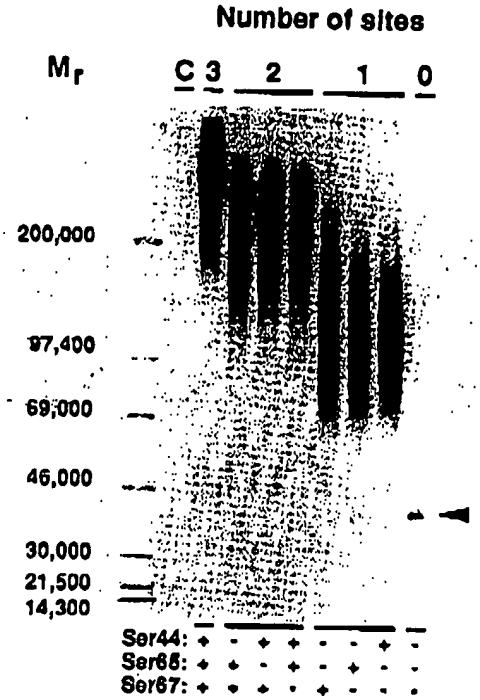

FIG. 4. **Identification of GAG acceptor sites.** Ryudocan GAG attachment sites were identified by analyzing L cell pools stably transfected with a wild-type ryudocan expression construction (pNWS144) or constructs containing Ser → Thr mutations (pNWS155–pNWS162, Fig. 1). Cultures were $Na_2{}^{35}SO_4$ labeled, tagged ryudocan was purified using 12CA5 monoclonal antibody, and immunoprecipitates were resolved by 5–12% linear gradient SDS-PAGE as described under "Experimental Procedures." The entire sample was loaded for *lanes C* and *0*, whereas 180,000 cpm was loaded for the remaining lanes. At this exposure, nontransfected L cell extracts (*C*) are devoid of 12CA5 reacting material. The sizes of ^{14}C-protein molecular weight markers are displayed. Apparent modal M_r was determined by detecting migration profiles with a Betascope 603 Blot Analyzer followed by standardizing against protein markers.

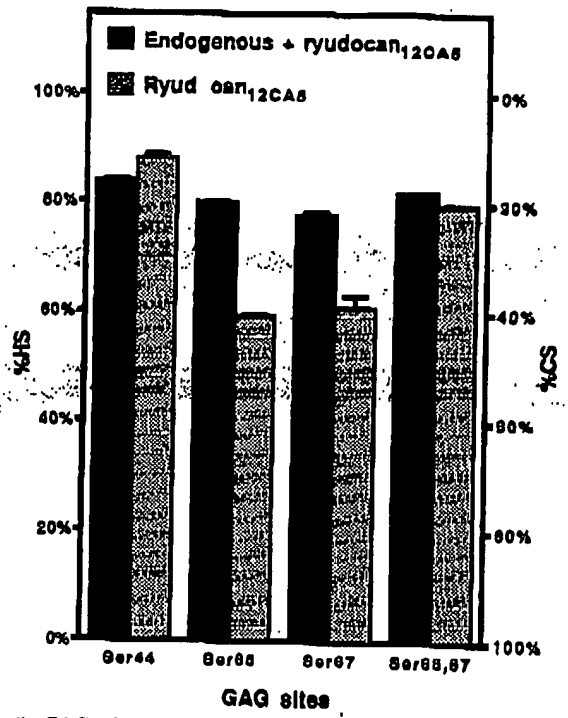

FIG. 5. **GAG chain attachment to individual acceptor sites.** Stable transfectants expressing epitope-tagged ryudocan molecules containing one or two GAG attachment sites were $Na_2{}^{35}SO_4$ labeled. For each sample, GAGs were obtained from both ryudocan$_{12CA5}$ (*stippled bars*) isolated by 12CA5 immunoprecipitation and total PGs (*black bars*) prepared by DEAE chromatography. Relative HS or CS content was enzymatically determined as described under "Experimental Procedures." Data from duplicate samples (mean ± range) are presented, and similar results were obtained in two additional experiments.

reduction in M_r of ~80,000. The molecular sizes of free GAG chains have been determined by SDS-PAGE, as outlined under "Experimental Procedures," and reveal that HS and CS exhibit a modal M_r of 53,000 and 22,000, respectively (all determinations were within 4% of mean, $n = 3$). The larger reduction in apparent M_r experienced by the PG, with stepwise elimination of acceptor sites, is probably because of the effect of GAG chains on the hydrodynamic radius of the PG or the amount of SDS bound by the PG. Regardless, these data demonstrate that ryudocan expressed in L cells exhibits three functional GAG acceptor sites and that, in most if not all molecules, all three sites are simultaneously occupied with GAGs.

Individual Ryudocan GAG Attachment Sites Are Functionally Similar—We next determined the extent to which HS or CS is attached to individual GAG acceptor sites by examining nonclonal populations that express ryudocan$_{12CA5}$ molecules possessing only a single acceptor site or possessing the Ser^{65,67} double acceptor site. The relative content of HS and CS was determined for ryudocan$_{12CA5}$ from each of the cell populations, whereas the total GAG fraction was utilized to standardize for potential variations between cell populations. The ^{35}S GAG incorporation was increased <20% in transfected cells as compared with nontransfected cells. Therefore, overexpression of ryudocan$_{12CA5}$ should not significantly bias the overall composition of the total GAG population.

The data show that HS and CS are attached to each single GAG acceptor site (Fig. 5). For each ryudocan$_{12CA5}$ species with a single GAG acceptor site, the relative content of HS or CS differs only slightly from the total GAG production of the cell population. Compared with the total GAG production, the attachment of GAGs at Ser^{44} is marginally biased toward HS; whereas the linkage of GAGs at Ser^{65} and Ser^{67} is marginally biased toward CS.

Functional independence of each acceptor site within intact ryudocan would imply that the single site preferences determined above should accurately predict the HS and CS content of the Ser^{65,67} double acceptor PG and wild-type Ser^{44,65,67} triple acceptor PG. This situation does not appear to be the case. The site biases of Ser^{65} ryudocan and Ser^{67} ryudocan predict that Ser^{65,67} ryudocan should have a relative HS content of 60%, whereas the observed level is 80% (Fig. 5). The site biases of Ser^{44} ryudocan, Ser^{65} ryudocan, and Ser^{67} ryudocan predict that wild-type Ser^{44}, Ser^{65}, Ser^{67} ryudocan of clone 19 should have a relative HS content of 69%, whereas the actual level is 87% (Fig. 3). The above data indicate that removal of two GAG acceptor sites slightly modifies the preference of HS and CS attachment at the remaining GAG acceptor site. Despite this minor alteration, the mutational analysis demonstrates that both HS and CS can be attached to each ryudocan acceptor site.

L Cells Produce Multiple Ryudocan Isoforms—The above results could have arisen by either homogeneous or heterogeneous glycanation of individual protein molecules. With homogeneous glycanation, cores could be partitioned at a specific frequency between HS-exclusive or CS-exclusive pathways, which would generate two types of homoglycans: a pure HS-ryudocan and a pure CS-ryudocan. Alternatively, HS or CS could be distributed at a set frequency for each single GAG acceptor site within a protein core. Such heterogeneous glycanation of a single core would produce a mixture of heteroglycans, isoforms with two HS chains/one CS chain and one HS chain/two CS chains, as well as homoglycans containing either three HS chains or three CS chains. To distinguish between

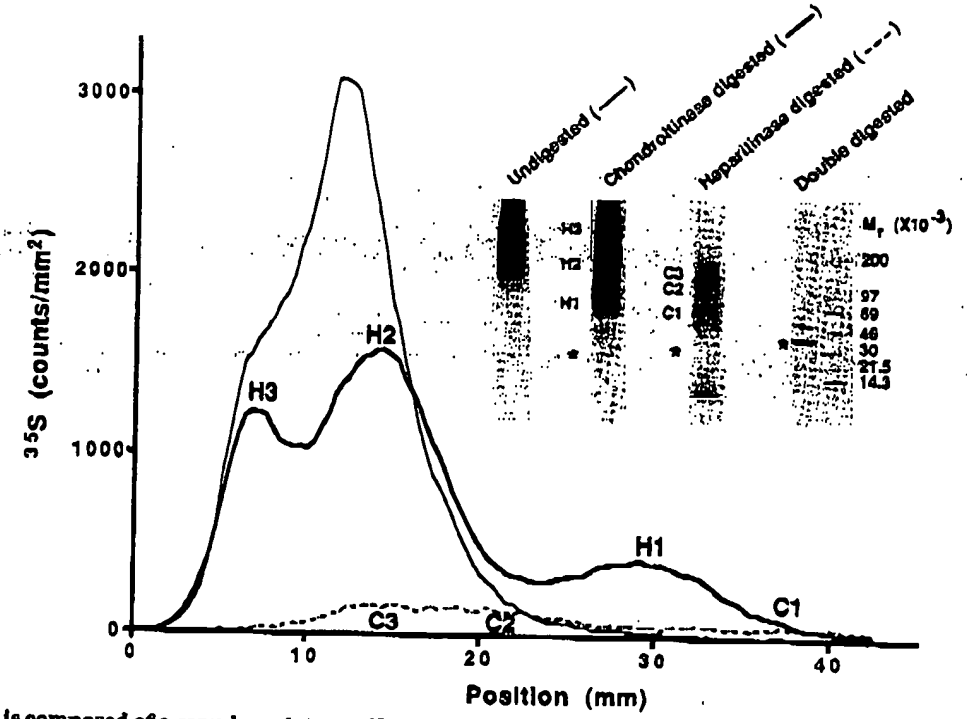

FIG. 6. **Ryudocan is composed of a complex mixture of homo- and heteroglycans.** Clone 19 was ^{35}S labeled, tagged ryudocan was isolated by immunoprecipitation, and samples containing 230,000 cpm were either untreated, digested with chondroitinase ABC or *Flavobacterium* heparitinase or both. Samples were then resolved using 3–15% SDS-PAGE and visualized by autoradiography (*inset*). Migration profiles were generated by direct Betascope digitization of dried gels as described under "Experimental Procedures." Instrument background was <10 counts/mm^2. Data were collected over 12 h and are displayed for untreated samples, *Flavobacterium* heparitinase-treated samples or chondroitinase ABC-treated samples. Sample migration was measured from the top of the resolving gel in 0.4-mm increments. M_r standards of 200,000, 97,400, and 69,000, respectively, migrated at 16.6, 33.4, and 39.3 mm. *Inset*, 3-day exposure autoradiograph. Sizes of ^{14}C-protein molecular weight standards are indicated. * indicates the position of minimally modified ryudocan while the species with M_r of ~300,000 is a copurified endogenous L cell protein. Analysis of ethanol-soluble radioactivity demonstrated that digestions were essentially complete. This conclusion was also confirmed by analysis of ryudocan digested with both enzymes (*inset*).

these two potential mechanisms, we examined by SDS-PAGE the size profiles of untreated and GAG lyase-treated ryudocan$_{13CA5}$ isolated from clone 19. This examination revealed the number and type of GAG chains attached to the protein core of various isoforms.

Given that the molecular size of HS is about twice that of CS, described above, homogeneous glycanation should produce a higher molecular weight pure HS-homoglycan and a lower molecular weight pure CS-homoglycan; thus, a bimodal distribution of wild-type ryudocan would result. Digestion of the wild-type ryudocan with *Flavobacterium* heparitinase would be expected to remove the high M_r component, whereas digestion of the wild-type ryudocan with chondroitinase ABC should destroy the low molecular weight species. Thus, treatment with either enzyme should generate simple unimodal distributions of differing molecular size. In contrast, heterogeneous glycanation should produce a roughly unimodal distribution of wild-type ryudocan because of the presence of a complex mixture of isoforms with differing ratios of HS to CS. Digestion of wild-type ryudocan with chondroitinase ABC should generate a trimodal distribution of core proteins with one, two, or three HS chains as well as the minimally modified core protein to which was attached three CS chains. The apparent molecular sizes of the HS containing core proteins should be equivalent to chondroitinase ABC-treated single acceptor, double acceptor, and triple acceptor core proteins (similar to Fig. 4). The digestion of wild-type ryudocan with *Flavobacterium* heparitinase should generate a reciprocal set of changes including production of the minimally modified core protein to which were attached three HS chains.

As shown in Fig. 6, wild-type ryudocan$_{13CA5}$ exhibits a roughly unimodal size distribution with an apparent modal M_r of about 280,000. Treatment with chondroitinase ABC results in a trimodal size distribution (Fig. 6, *Chondroitinase-digested*). This effect is not caused by proteolytic degradation since cleavage was not observed when chondroitinase ABC was incubated with [^{3}H]leucine-labeled ryudocan$_{13CA5}$ core protein (results not shown). Curve fitting analysis reveals that the observed HSPG profile can be duplicated by three overlapping Gausian curves with modal M_r of 270,000 (H3), 240,000 (H2), and 120,000 (H1), respectively. The H3 species represents the largest ryudocan isoform, whose molecular size is not affected by chondroitinase ABC treatment. The H2 and H1 peaks comigrate with chondroitinase ABC-treated double and single acceptor site ryudocan$_{13CA5}$, respectively (results not shown). We also note a band with an apparent M_r of 39,000, which comigrates with minimally modified ryudocan. Given that wild-type ryudocan exhibits three GAG acceptor sites that are fully occupied, we conclude that H3 represents an isoform that contains three HS chains, that H2 is derived from isoforms that possess two HS chains and one CS chain (H2C1), that H1 is produced from isoforms that contain one HS chain and two CS chains (H1C2), and that the minimally modified core protein is generated from an isoform that exhibits three CS chains (C3).

The digestion of wild-type ryudocan$_{13CA5}$ with *Flavobacterium heparitinase* provides complementary data. As noted in Fig. 6, (*Heparitinase-digested*) the CS-bearing PGs generated by the above treatment resolve into two peaks with modal M_r of 160,000 (C3/C2) and 85,000 (C1), respectively. This effect is not caused by proteolytic degradation since cleavage was not observed when *Flavobacterium* heparitinase was incubated with [^{3}H]leucine-labeled ryudocan$_{13CA5}$ core protein (results not

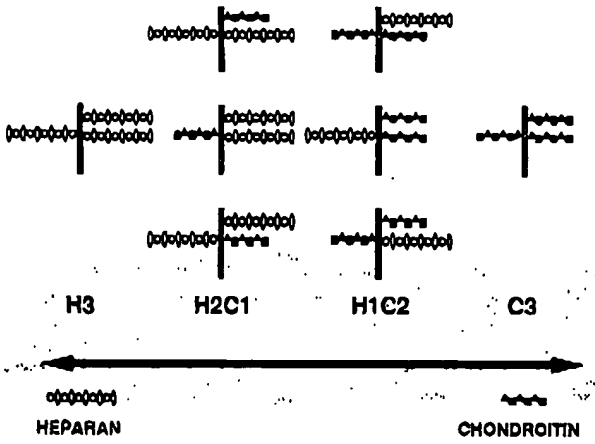

FIG. 7. **Schematic representation of ryudocan isoforms.** Displayed are all eight possible ryudocan isoforms synthesized by L cells. The ryudocan core protein always contains three GAG chains. HS chains (*open*) are twice as long as CS chains (*closed*).

shown). The broad C3/C2 and C1 peaks comigrate with *Flavobacterium* heparitinase-treated triple/double acceptor site ryudocan$_{12CAS}$ and single acceptor ryudocan$_{12CAS}$, respectively (results not shown). The C3 and C2 peaks exhibit less discrimination than the H3 and H2 peaks because of the smaller molecular size of CS. We also note a band with an apparent M_r of 39,000, which comigrates with minimally modified ryudocan. Given that wild-type ryudocan exhibits three GAG acceptor sites that are fully occupied, we conclude that C3 represents a form that contains three CS chains, that C2 is derived from isoforms that possess two CS chains and one HS chain (C2H1), that C1 is produced from isoforms that contain one CS chain and two HS chains (H1C2), and that the minimally modified core protein is generated from the isoform that exhibits three HS chains (H3).

We have also performed this entire analysis on endogenous ryudocan immunopurified from extracts of nontransfected L cells by intracellular domain-specific or ectodomain-specific antiserum. For either preparation, endogenous ryudocan digestion profiles mimicked the above described patterns elicited from ryudocan$_{12CAS}$ (results not shown). These data further confirm that ryudocan$_{12CAS}$ is representative of the endogenous PG.

The combined data strongly indicate that ryudocan is synthesized as a mixture of the following isoforms: pure HS-ryudocan (H3), heteroglycans (H2C1 and H1C2), and pure CS-ryudocan (C3) (Fig. 7). The above results confirm the mutational analysis of ryudocan$_{12CAS}$, which indicates that individual GAG acceptor sites can bear either HS or CS and strongly support heterogeneous glycanation of individual core proteins.

L Cells Also Produce Pure Homoglycans—The heteroglycan-like nature of ryudocan could be an innate property of the core protein, or L cells might only be able to generate heteroglycans. To exclude the latter possibility, we examined endogenous L cell PGs to identify core proteins that are pure CS- or HS-homoglycans. In the first approach, [^{35}S]methionine-labeled PGs were partially purified from cell extracts by ultrafiltration to remove material <100,000 kDa, followed by DEAE chromatography, as described under "Experimental Procedures." These PGs were then subjected to GAG lyase treatment and SDS-PAGE analysis (Fig. 8A). Two core proteins (apparent M_r = 130,000 and 52,000) appeared with chondroitinase ABC digestion. The intensity of the bands was not increased by simultaneous treatment with *Flavobacterium* heparitinase. The two core proteins were not detected when PGs wer only digested with *Flavobacterium* heparitinase. Similar results were also obtained when

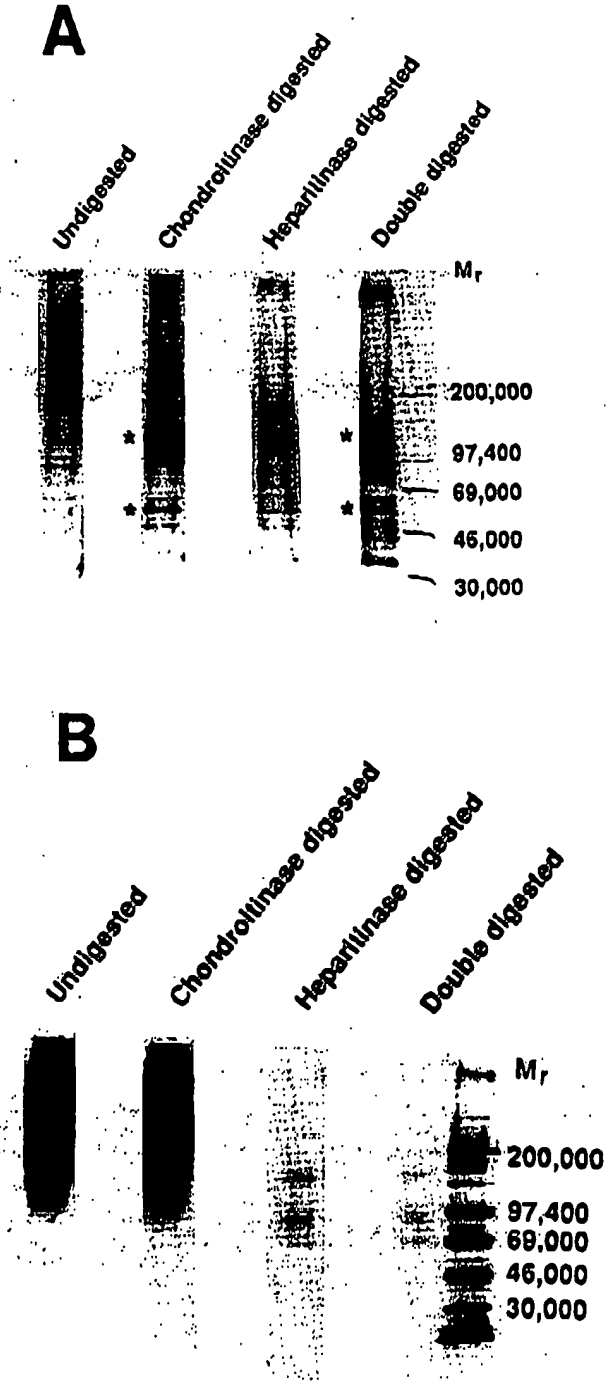

FIG. 8. **Certain endogenous L cell proteoglycans are pure homoglycans.** Homoglycans were identified using [^{35}S]methionine-labeled partially purified PGs (*panel A*) or $Na_2{}^{35}SO_4$-labeled immunoprecipitated glypican (*panel B*) isolated from L cells. Samples (18,000 cpm) were untreated or digested with chondroitinase ABC and/or *Flavobacterium* heparitinase; reactions were then resolved by 8–16% linear gradient SDS-PAGE and visualized by fluorography. Sizes of ^{14}C-labeled molecular weight standards are indicated. *Panel A*, PGs were purified by DEAE chromatography and ultrafiltration as described under "Experimental Procedures." * indicates the position of M_r = 130,000 and 52,000 protein cores from pure CS-homoglycans. Note that GAG lyase treatment does not degrade cross-contaminating non-PG species. *Panel B*, each lane was loaded with material derived from an initial 7,000 cpm. A 2-month exposure is presented to demonstrate the complete absence of CS-glypican.

partially purified PGs were labeled with [^{3}H]leucine and digestions with GAG lyases were carried out as outlined above (data not shown). Together, these data demonstrate that L cells are able to produce pure CS-homoglycans.

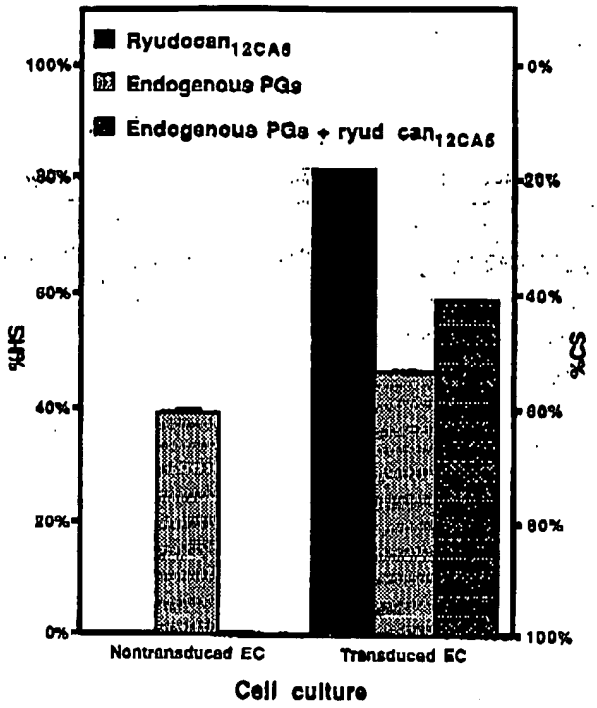

FIG. 9. **Ryudocan is a hybrid proteoglycan in endothelial cells.** ^{35}S-Labeled GAGs were prepared from early passage endothelial cell PGs (*Nontransduced EC*) and from extracts of ryudocan-transduced cells (*Transduced EC*) that were fractionated into total PGs (*cross-hatched bars*), ryudocan$_{12CA5}$ (*black bars*), and PGs depleted of ryudocan$_{12CA5}$ (*stippled bars*), described under "Results." The GAG composition was then enzymatically determined as outlined under "Experimental Procedures." Data from duplicate samples (mean ± range) are presented, and similar results were obtained in two additional experiments.

In the second approach, metabolically labeled glypican, which has been identified in other cell types as an HS-homoglycan (55, 56), was isolated from L cells by immunopurification with an antiserum raised against a glypican fusion protein. This antibody has been used previously to isolate glypican by immunoprecipitation (41). The SDS-PAGE analysis of metabolically labeled glypican showed complete degradation with *Flavobacterium* heparitinase and no alteration with chondroitinase ABC (Fig. 8*B*). Furthermore, detailed examination by Betascope of the SDS-PAGE patterns revealed that chondroitinase treatment did not alter the size distribution of metabolically labeled glypican. Together, the results demonstrate that glypican is a pure HS-homoglycan. The existence of pure CS- and HS-homoglycans provides strong evidence that the heteroglycan nature of ryudocan is an inherent property of the protein core rather than a consequence of synthesis in L cells.

Ryudocan Is a Hybrid Proteoglycan In Endothelial Cells—To determine whether cell type affects the GAG composition of ryudocan, we expressed ryudocan$_{12CA5}$ in early passage endothelial cells. Overexpression was accomplished by subjecting endothelial cells to two transduction cycles with a replication-defective retroviral vector. This process increased ^{35}S incorporation into total GAGs by 1.7-fold (data not shown) and altered the composition of synthesized GAGs. Fig. 9 demonstrates that the HS content of total PGs increased from 40% (nontransduced cells; endogenous PGs) to 60% (transduced cells; endogenous + ryudocan$_{12CA5}$). To investigate the mechanism for this elevation in HS synthesis, we examined 12CA5-purified ryudocan$_{12CA5}$ as well as endogenous PGs obtained from extracts depleted of ryudocan$_{12CA5}$ by two immunoprecipitation cycles with 12CA5 (this procedure removes ~75% of tagged ryudocan). Compared with the total PG population, the HS content of ryudocan$_{12CA5}$ is higher, whereas the HS level of endogenous PGs is lower. Furthermore, the HS content of endogenous PGs in transduced endothelial cells is almost identical to that of total PGs in nontransduced endothelial cells. Thus, expression of ryudocan$_{12CA5}$ in endothelial cells does not alter the composition of endogenous PGs. The elevated levels of HS in transduced endothelial cells result from an overexpression of HS-rich ryudocan. Most importantly, ryudocan is synthesized in endothelial cells (which predominantly produce CS) as an HS-rich hybrid proteoglycan, similar to L cell-produced ryudocan.

DISCUSSION

We have determined the relative amounts of HS and CS attached to each of the GAG acceptor sites of ryudocan. Our investigation was facilitated by expressing a ryudocan form containing the epitope for the 12CA5 monoclonal antibody (44). Previous investigators have employed epitope tagging to isolate recombinant proteins overexpressed in yeast (43, 57) or to localize proteins expressed in yeast and higher eukaryotic cells (58, 59). To our knowledge, this study represents the first use of this approach to characterize biochemically a protein synthesized in higher eukaryotic cells. This analysis was made possible by labeling GAGs to high specific activity with $Na_2{}^{35}SO_4$ and should be applicable to the detailed examination of cell specific modifications of other PGs.

Our study revealed that ryudocan possesses three GAG attachment sites that are always occupied and that each site bears similar proportions of either HS or CS. We wondered whether the three GAG acceptor sites on ryudocan are randomly occupied by HS or CS, and whether they function in a completely independent manner. The observation that the relative proportions of HS and CS attached to acceptor sites is somewhat affected by the number of acceptor sites per core protein (Fig. 5) suggests that these regions may not act in a completely independent manner. The combined effects of GAG acceptor site occupancy bias and dependent function of acceptor sites can be assessed from the distribution of ryudocan isoforms. If each site is characterized by unbiased GAG chain occupancy and acts in a completely independent fashion, then the abundance of each isoform class (H3, H2C1, H1C2, C3) will follow a binomial distribution, which can be calculated from the relative molar ratios of HS and CS attached to ryudocan. Both GAG acceptor site occupancy bias and dependent function of acceptor sites should produce deviations from the binomial distribution.

The relative abundance of the different ryudocan isoforms can be determined from the SDS-PAGE patterns generated by digestion of ryudocan$_{12CA5}$ with GAG lyases. We have employed a Gaussian curve fitting routine to obtain the total radioactivity present in peaks H3 to H1 and C3 to C1. The molar ratio of HS-bearing ryudocan species was determined by correcting the total radioactivity in peaks H3, H2, and H1 for chain number, which then provides the relative amounts of H3, H2C1, and H1C2. Similar analysis of peaks C1, C2, and C3 allows us to obtain the relative amounts of H2C1, H1C2, and C3, respectively.[4] The relative amounts of all isoform classes can be calculated because H2C1 and H1C2 are common to both determinations. Based upon the above analysis, the relative abundance of isoforms is 20% H3, 33% H2C1, 34% H1C2, and 13% C3. From the above distribution, we calculate that the molar abundance of HS on ryudocan is 53%. Alternatively, given that ryudocan possessed 87% of GAG ^{35}S as HS (Fig. 3), that HS was

[4] Discrimination of the C3 and C2 components of the C3/C2 peak was accomplished by subtracting the C2 component of *Flavobacterium* heparitinase-digested double acceptor ryudocan$_{12CA5}$.

2.4-fold larger than CS (described above), and that HS incorporated 2.6-fold more ^{35}S than CS (data not shown), we calculate the molar abundance of HS chains on ryudocan as 57%, which is in excellent agreement with the previous estimate. Based on an average value of 55%, the binomial expansion predicts an isoform distribution of 17% H3, 41% H2C1, 33% H1C2, and 9% C3. The empirical and theoretical calculations generate similar, but not identical, distributions, which suggests that at most, a very minor degree of occupancy bias for each acceptor site and/or functional dependence between acceptor sites may be operable during glycosylation.

The promiscuity of ryudocan GAG attachment sites is an inherent property of the core protein (Figs. 8 and 9). A similar promiscuity of GAG acceptor sites has been suggested for the serglycins, secretory granule PGs that possess up to 24 consecutive, heavily substituted, Ser-Gly repeats (60, 61). The mucosal mast cell PG bears CS (17), the connective tissue mast cell PG contains heparin (16), and the rat basophilic leukemia form possesses both heparin and CS (18). However, the specificity of the individual GAG attachment sites is unknown, as only about half of the potential acceptor sites are endowed with GAGs (62, 63). Other hybrid PGs have been identified (22, 64, 65), but the occupancy bias and functional dependence of GAG acceptor sites in these species have yet to be determined.

How does the glycosylation machinery distinguish among HS-limited, CS-limited, and HS/CS-permissive GAG acceptor sites? Perusal of the immediate primary sequence that flanks putative and known acceptor sites from several CSPGs and HSPGs fails to reveal any consistent distinguishing feature (for review see Ref. 1). The detailed mutagenesis of the single acceptor site on decorin also argues strongly against a simple consensus sequence (12). Consistent with this conclusion, the acceptor sites on ryudocan (underlined) are functionally similar but have a dramatically different primary sequence (RYFSGALP *versus* FELSGSGDLD). Indeed, the immediate primary structure around an acceptor site is unlikely to convey specificity because the multiple consecutive acceptor sites of serglycin are likely to be promiscuous. Given the lack of any distinguishing features between HS and CS acceptor sites, it is most probable that specificity, with respect to the attachment of GAG chains, is determined by distant sequences and/or the higher order structure of the core protein.

Ryudocan is a member of the syndecan family, and all such members exhibit very distinct extracellular domains but possess extremely homologous transmembrane and intracellular regions. Syndecan, the prototypical member, may well contain promiscuous sites since the amino-terminal portion contains both HS and CS. Additionally, two CS-exclusive sites occur in the carboxyl-terminal region (66). In contrast, we have demonstrated that glypican, from L cells, is a pure HSPG. Glypican, unlike syndecan family members, attaches to the cell membrane via a covalently linked glycosylphosphatidylinositol anchor (55). It is tempting to speculate that interactions between the glycosylation machinery and the conserved transmembrane/intracellular domains may create hybrid PGs by facilitating the attachment of CS to potentially promiscuous acceptor sites. We are presently testing this hypothesis by domain swapping experiments between glypican and ryudocan.

The production of multiple isoforms of ryudocan may serve to expand the functional diversity of this cell surface component and other heteroglycans. It has been suggested that syndecan family members participate in many different biologic processes including regulation of blood coagulation, cell adhesion, maintenance of cell morphology, and signal transduction. The ability of these components to act in this diverse fashion appears to be predominantly a result of the covalently attached HS chains, which bind protease inhibitors, cell adhesion molecules, peptide growth factors, circulating lipoproteins, and lipolytic enzymes (for review see Ref. 22). The attachment of different numbers of HS and CS chains to syndecan family members may alter interactions with specific proteins and hence modify the biologic function of these components (67, 68). In this regard, we have noted that the distribution of ryudocan isoforms is altered when cells shift from the exponentially growing to the postconfluent state.[6] This observation could represent an important linkage between growth state, the structure of PGs, and the function of these molecules.

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